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14. ABSTRACT

As an approach to identify potential Shh-responding stem cells in the mouse prostate, we used Genetic Inducible Fate Mapping (GIFM) to follow the fate of Shh-responding cells both during prostate development and during androgen-mediated regeneration of the gland in the adult, two processes that are driven by stem or progenitor cell expansion. As *Gli1* expression is a sensitive readout of Shh signaling, we used a *Gli1*$_{CreER}$ allele and *Rosa26* reporter to fate map Shhresponding cells. We show that Shh-responding cells do not expand over time in the normal homoeostatic prostate, but these same cells do expand massively after androgen-mediated regeneration, indicating that Shh-responding cells are normally quiescent, but retain the ability to expand in the adult prostate. The expansion of cells is confined to stromal fibroblasts and smooth muscle cells; no glandular epithelial cells are marked. These results indicate that *Gli1* either specifically marks stromal stem cells that expand during regeneration to give rise to the two stromal cell types, or that fibroblasts and smooth muscle cells in general have a high capacity for proliferation even in the adult prostate. To determine whether the marked Shh-responding cells have the capacity for selfrenewal, we subjected *Gli1*$_{CreER}$*; Rosa26* mice to eight cycles of prostate involution and regeneration. Cells marked before castration expand after 8 cycles of involution/regeneration, indicating that the initially marked Shh-responding cells are self-renewing. Additionally, using *Gli1* null mutant mice, we demonstrate that *Gli1* is required to drive stromal expansion during prostate regeneration. Based on our results, we propose a model wherein Shh is expressed in adult prostate epithelial cells, the signal is received by the adjacent stroma, which responds by expressing critical genes, including the transcription factor *Gli1*, that result in expansion of the two stromal cell types.

15. SUBJECT TERMS
Prostate, Shh, Gli1, Stem Cells, Mouse

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Introduction

The specific aims for the approved proposal are as follows:

I. Determine the fate of Shh-responding cell populations in the mouse prostate, using *Gli1-CreER* mice and GIFM.

Hypothesis: Shh directly regulates expansion of the glandular stroma but not the epithelial compartment during development, normal adult homeostasis and regeneration of the prostate.

II. Ascertain whether *Gli1* is required to drive expansion of the stromal compartment of the prostate using GIFM during development and androgen-mediated regeneration of the prostate in *Gli1* mutant mice.

Hypothesis: Gli1 is required to drive stromal cell expansion.

III. Assess the fate of Shh-responding cells during progression of prostate cancer and the role of *Gli1* in prostate cancer, using GIFM and *Gli1* mutant mice with the TRAMP and other prostate cancer models.

Hypothesis: The epithelium becomes Shh-responsive during malignant transformation in prostate cancer and Shh-responding cells are enriched in metastatic tumors. Furthermore, *Gli1* null mutant prostates will form fewer and/or less aggressive tumors.

IV. Determine whether there are populations of normal adult stem cells and/or cancer stem cells in the mouse prostate that respond to Shh, using clonal analysis and Florescence Automated Cell Sorting (FACS)**.**

Hypothesis: Stromal stem cells in the mouse prostate respond to Shh signaling, and epithelial cells that aberrantly respond to Shh signaling represent cancer stem cells.

The experiments pertaining to specific aims I and II are nearly complete. We are in the process of compiling data from these experiments to prepare a manuscript to submit for publication. Additionally, the mice required to conduct the experiments for specific aims III and IV have been generated, and we are waiting for these mice to mature to the appropriate ages to conduct the proposed experiments for these specific aims.

Body

Task 1. To determine the fate of Shh-responding cell populations in the mouse prostate (months 1-12).

a. Genetic Inducible Fate Mapping (GIFM) studies during embryonic and early postnatal development, during normal adult homeostasis and during androgen mediated regeneration in the adult (n=6 animals for each experiment)
    a. Tamoxifen gauvage for development and homeostasis experiments
    b. Castration, androgen pellet implantation and tamoxifen gauvage for regeneration experiments.
b. Sectioning prostates from GIFM experiments and marker staining to determine which cell types are marked.
c. BrdU injections, TUNEL and Caspase staining to determine which cell types are proliferating and which cell types are dying during regeneration.

Progress:

GIFM studies of Gli1-expressing cells during early postnatal development and during androgen-mediated regeneration in the adult are complete (figure 7). We have also nearly completed a similar analysis of Shh-expressing cells to complement our proposed studies. We originally proposed to mark Shh-responding cells during regeneration by gavaging with tamoxifen at the same time as initial androgen administration. We have changed the strategy for marking these cells, reasoning that any Shh-responding stem cells that expand during regeneration would likely be marked in the normal homeostatic prostate as well. Therefore, we reasoned that by gavaging Gli1-CreER ; R26R reporter mice *before* castration should yield the same expansion. Our results from these experiments indicate that, in fact, Shh-responding cells marked before castration, expand markedly after regeneration (figure 4). Regarding the homeostasis experiments, we have analyzed several animals two weeks and one year after tamoxifen gavage, and we will begin the analysis of the final set of animals, gavaged one year ago, in the next several weeks (figure 2). This will bring the total n to 6 animals per group.

Using cell type specific markers, we have determined conclusively that marked cells after development and regeneration are smooth muscle cells and not epithelial cells (figure 5). We are currently trying to show that some of the marked cells are fibroblasts as well, but we have encountered difficulty

determining the proper conditions for the fibroblast specific antibodies (Vimentin). We hope to resolve this issue in the next few months.

Preliminary BrdU and Caspase staining indicates that there are dividing cells in the stromal and in the epithelial compartments during development and regeneration. We are currently assessing whether the marked cells (cells that are derived from the Gli1-lineage) are dividing proportionally more or less than non-marked stromal cells. Caspase staining indicates that there is cell death in both compartments during involution, following castration (figure 3), and we are currently determining what proportion of marked cells undergo cell death, relative to non-marked stromal cells.

Task 2. Ascertain whether *Gli1* is required to drive expansion of the stromal compartment of the prostate (months 10-18).

a. GIFM studies, using *Gli1* mutant mice during embryonic and early postnatal development, during normal adult homeostasis and during androgen mediated regeneration in the adult. (n=6 animals for each experiment)
    a. Tamoxifen gauvage for development and homeostasis experiments
    b. Castration, androgen pellet implantation and tamoxifen gauvage for regeneration experiments.
b. Sectioning prostates from GIFM experiments and marker staining to determine which cell types are marked.
c. BrdU injections, TUNEL and Caspase staining to determine which cell types are proliferating and which cell types are dying during regeneration.
d. FACS analysis using stromal cell markers of *Gli1* wildtype and *Gli1* null mutants after regeneration to quantify reduced stroma phenotype

Progress:

GIFM studies on late embryonic development and adult regeneration, using *Gli1* mutant mice are nearly complete. We changed the marking strategy in the same manner as described above for task 1. When we mark Gli1-expressing cells during postanatal development and follow their fate throughout development, we see less expansion than in wild-type mice. We are presently confirming these findings with additional mice. Furthermore, after regeneration, we see less overall stroma than in wild-type regenerated prostates as well as fewer marked cells (figure 8). Preliminary BrdU and Caspase staining reveals less proliferation and more cell death in the stromal compartment in *Gli1* mutant mice, as compared to the stromal compartment of wild-type mice. We are in the process of confirming these results with additional mice.

I recently used a portion of the travel budget to travel to the laboratory of Dr. Wade Bushman, at the University of Wisconsin at Madison to learn the techniques of prostate single cell suspensions to make “prostaspheres” and for FACS analysis. We are currently in the process of implementing these protocols in the lab in order to quantify the reduced stroma phenotype in the *Gli1* mutant mice.

Task 3. Assess the fate of Shh-responding cells during progression of prostate cancer and the role of *Gli1* in prostate cancer (months 1-36).

a. Breed *Gli1*^{CreER} mice on to a C57 background (months 1-12).
b. Breed *Gli1*^{CreER} *; R26R* mice with *TRAMP* mice (months 1-18)
c. Breed *Gli1*^{nLacZ} mice on to a C57 background and then to the TRAMP mice (months 1-18)
d. GIFM studies with *Gli1*^{CreER} *; R26R*; *TRAMP* mice (months 12-36)
    a. Tamoxifen gauvage before onset of tumorigenesis.
    b. Tamoxifen gauvage after tumorigenesis, before metastasis.
    c. Marker analysis of tumor tissue from a. and b. to determine whether marked cells contribute to primary tumors and metastatic tumors.
e. Breed *Gli1* null mutants with *TRAMP* mice (months 1-12).
    a. Analysis of tumor number and metastasis

<u>Progress:</u>

Preliminary analysis of the first generation of TRAMP mice crossed to outbred mice indicates that these mice develop tumors with the same frequency as TRAMP mice on a C57/Bl6 inbred background. Therefore, we have decided to conduct our analysis on mixed C57/Bl6 and Swiss Webster outbred mice. These breedings are on schedule, and we plan to begin our initial analysis in the next month. In-situ hybridization experiments on tissue sections of metastatic tumors from various organs, taken from TRAMP mice, indicate increased expression levels of Shh, and Ihh. Additionally, we observe expression of *Gli1* in epithelial cells in these tumors.

Task 4. Determine whether there are populations of normal adult stem cells and/or cancer stem cells in the mouse prostate that respond to Shh (months 12-36).

a. Clonal analysis of Shh-responding cells in the regenerating prostate (months 12-36) (n=6 animals for each experiment)
    a. Involution/regeneration experiments described in task 1, with low-dose of tamoxifen
b. FACS sorting and marker analysis of tumor cells to identify epithelial cancer stem cells.

Progress:

As an additional test to determine if the Gli-expressing cells marked before castration are self-renewing stem cells, we have gavaged several mice with tamoxifen and then subjected these mice to more than 12 rounds of involution and regeneration. We reasoned that if these marked cells are true self-renewing stem cells, then the expansion should be maintained after several cycles of involution and regeneration. Initial analysis of mice cycled eight times indicates that the expansion of marked stromal cells is maintained after multiple cycles.

Key Research Accomplishments

- Gli1 marks a population of stromal stem cells in the adult mouse prostate
    - Gli1-expressing cells, marked before castration, expand massively after regeneration, in the stroma and not in the epithelium.
    - Gli-expressing stem cells self renew, as indicated by the maintenance of this expansion after multiple rounds of involution/regeneration
    - Gli1-expressing cells are normally quiescent (slow cycling), as evidenced by the lack of expansion seen in the normal homeostatic prostate, analyzed one year after tamoxifen gavage.
- Gli1 marks a population of stromal stem cells in the developing prostate
    - Gli1-expressing cells marked during embryonic and postnatal development expand during development, in the stroma
- Stromal stem cell expansion of Gli1-expressing cells is dependent on *Gli1*
    - GIFM of Gli1-expressing cells, in *Gli1* mutants, show little or no expansion during development and during regeneration in the adult.
    - Overall stromal compartment is reduced after regeneration in *Gli1* mutant mice.

Reportable Outcomes

Abstracts:

Levine CM and Joyner AL: Genetic inducible Fate Mapping Uncovers the Behavior of Shh-Responding Cells in the Prostate.
(Poster presented at Building a Better Mouse II conference, July 2007, Nashville, TN)

The mammalian prostate is derived from two cellular lineages: an endodermally derived glandular epithelium and a mesodermally derived stroma composed of fibroblasts and smooth muscle cells. The secreted factor Sonic Hedgehog (Shh) is first expressed by urogenital sinus epithelial cells at E16.5. In response to Shh, the adjacent urogenital mesenchyme expresses *Gli1*, a transcriptional target of Shh signaling. As an approach to study the behavior of Shh-responding cells in the prostate, we used Genetic Inducible Fate Mapping (GIFM) to follow the fate of Shh-responding cells both during postnatal development and during androgen-mediated regeneration of the gland in the adult, two processes that are driven by stem or progenitor cell expansion. As *Gli1* expression is a sensitive readout of Shh signaling, we used a *Gli1*^{CreER} allele and *Rosa26* reporter (Ahn & Joyner, Cell, 2004) to fate map Shh-responding cells. We show that the few stromal cells that are marked initially by GIFM at P14 or in the adult expand greatly during subsequent development (P14-P28) or during androgen-mediated regeneration, respectively. In both cases, the expansion of cells is confined to stromal fibroblasts and smooth muscle cells; no glandular epithelial cells are marked. These results indicate that *Gli1* either specifically marks stromal stem cells that expand during development and regeneration to give rise to the two stromal cell types, or that fibroblasts and smooth muscle cells in general have a high capacity for proliferation even in the adult prostate. Furthermore, using *Gli1* null mutant mice, we demonstrate that *Gli1* is required to drive stromal expansion during prostate regeneration. Based on our results, we propose a model wherein Shh is expressed in adult prostate epithelial cells, the signal is received by the adjacent stroma, which responds by expressing critical genes, including the transcription factor *Gli1*, that result in expansion of the two stromal cell types.

Presentations:

New York University School of Medicine, 2008 MSTP retreat: Stem Cells in the Mouse Prostate Stroma Respond to Shh.

Conclusion

In the first year of funding for this project, we have nearly completed the first two specific aims of the proposal. We have demonstrated that stromal stem cells in the normal adult prostate and in the developing prostate respond to Shh-signalling. We have evidence that these Shh-responding stem cells are normally quiescent, expand rapidly and massively during regeneration and development and that these cells are self-renewing. We have also demonstrated that the expansion of these Shh-responding stem cells is dependent on the transcription factor Gli1. We plan to determine whether these self are multipotent (i.e. give rise to both smooth muscle cells and fibroblasts) using clonal analysis.

Additionally, we have begun our analysis of Shh-responding cells in tumor initiation, progression and metastasis. Preliminary analysis of metastatic tumors from TRAMP mice indicates that Shh, Ihh and Gli1 are all expressed at high levels in the epithelial cells of prostate cancer metastases. In the next year of funding, we plan to use GIFM to determine whether it is the same Shh-responding stromal stem cells in the normal adult prostate that undergo transformation and contribute to the epithelial tumors that develop in TRAMP mice.

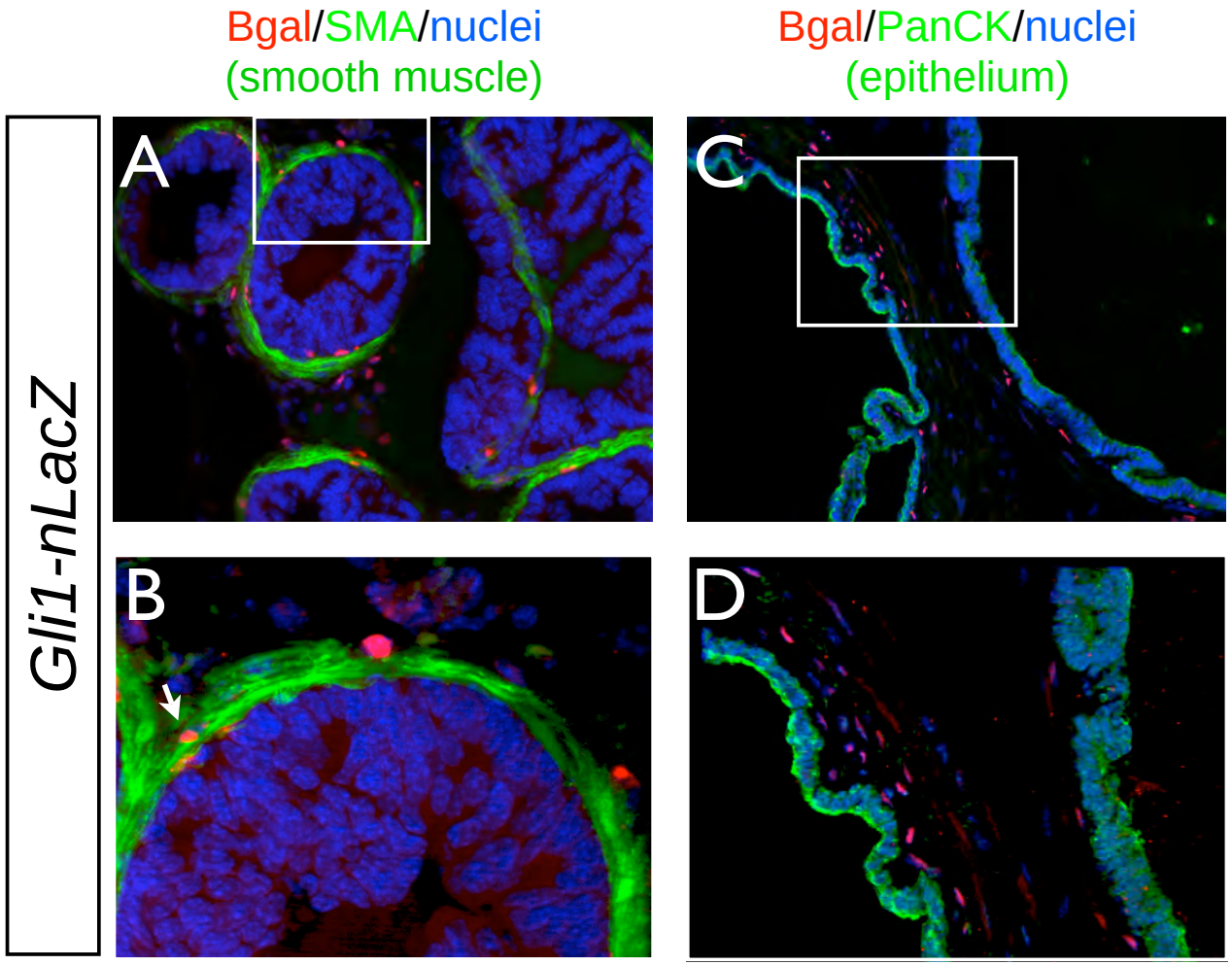

Figure 1. Gli1-expressing cells in the adult mouse prostate are confined to the stroma and not the epithelium. Sections from dorsal prostate of 2 month-old Gli1-LacZ mice, analyzed using antibodies against B-gal, smooth muscle actin (smooth muscle cells) and PanCytokeratin (epithelial cells). Marquis in A and C, indicates region magnified in B and D. Arrow in B, indicates double labeled cell.

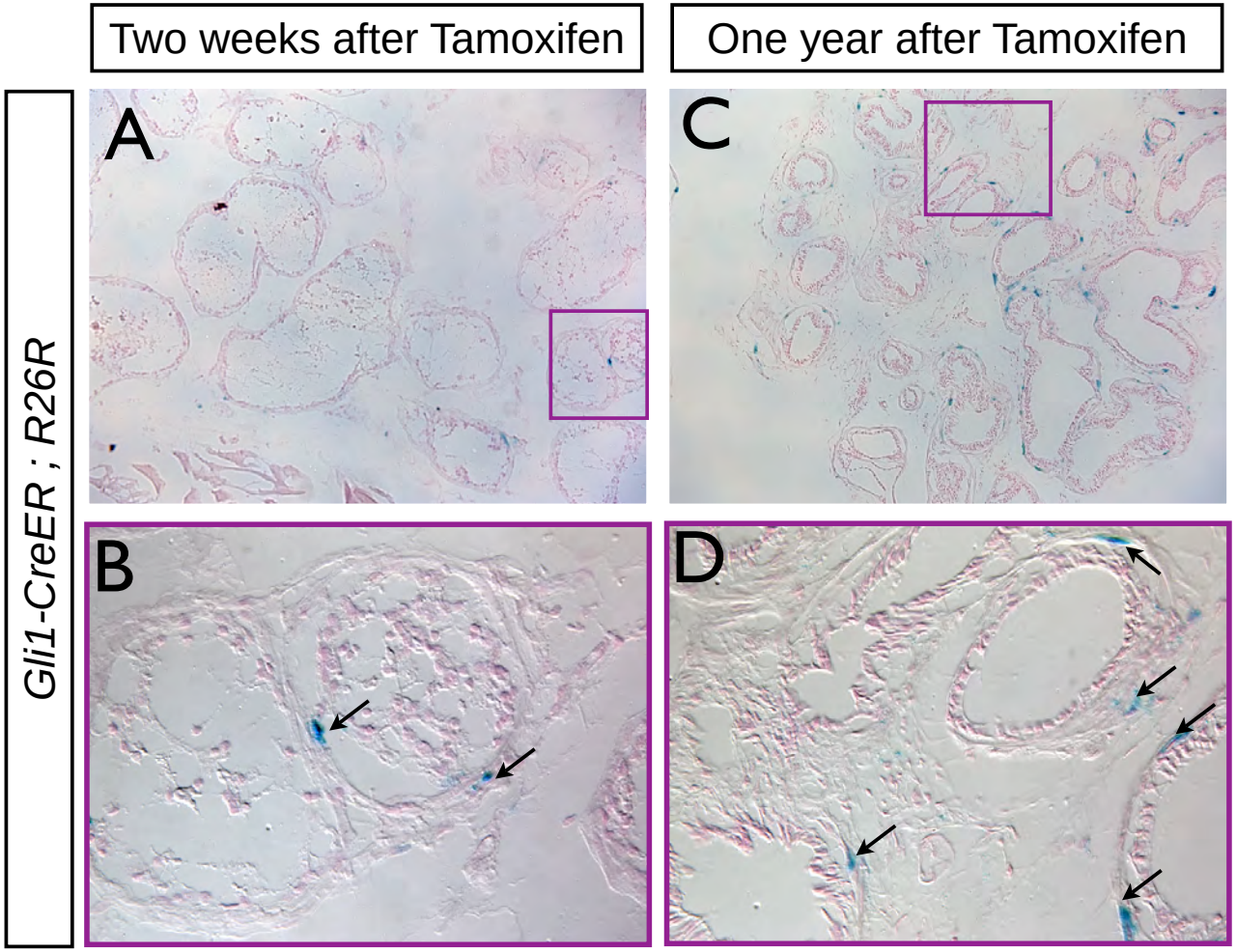

Figure 2. Gli1-expressing cells in the prostate are maintained for over a year in the stroma. 2-month old Gli1-CreER ; R26R/R26R micer were given taxmoxifen. Prostates sections from the dorsal prostate were analyzed by X-gal staining 2 weeks and 1 year later. Marquis in A and C, indicate regions of higher magnification in B and D. Arrows in B and D indicate marked cells.

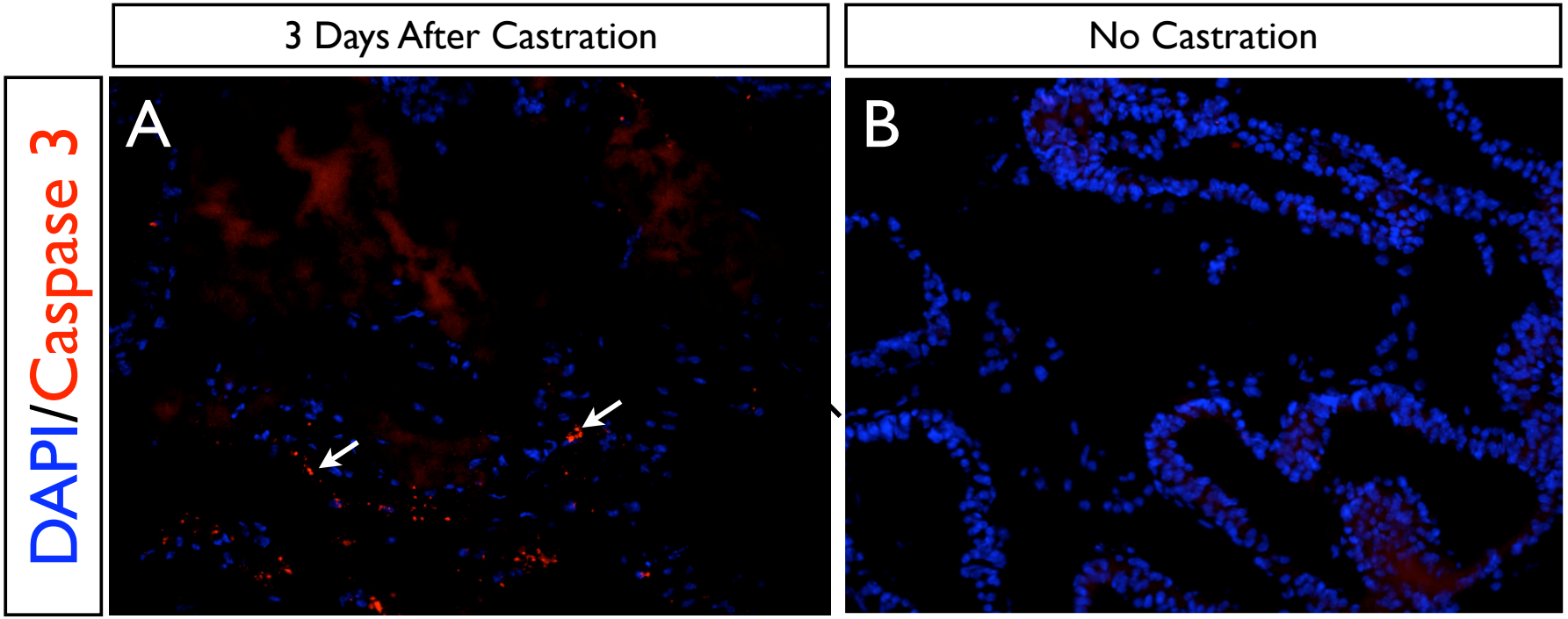

Figure 3. Stromal cell death during involution. 2 month-old wildtype mice were castrated and sacrificed 3 days later. A. Sections from the dorsal prostate were analyzed for cell death, using an antibody against activated Caspase-3. B. Section from non-castrated age-matched control mouse. Arrows in A, indicate Caspase-3 positive cells.

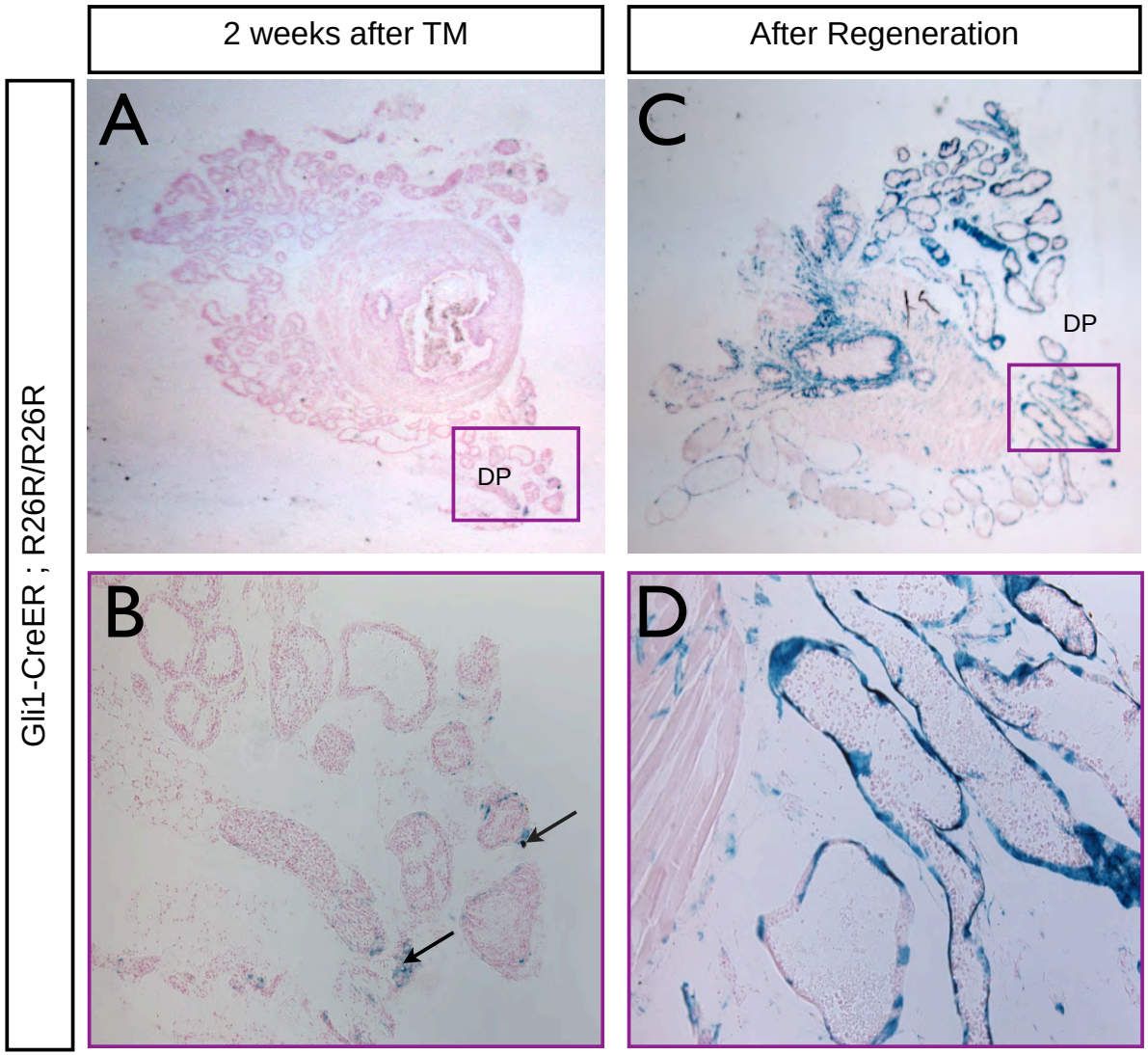

Figure 4. *Gli1* derived cells contribute to the prostatic stroma during androgen mediated regeneration after castration-induced involution. Two month old *Gli1*$^{CreER/+}$; R26R/R36R were given tamoxifen and castrated 2 weeks alter. After a two-week involution period, subcutaneous androgen pellets were implanted. Prostate sections were analyzed by X-gal staining. Marquis in A and C, indicate regions of higher magnification shown in B and D. Arrows in B indicate marked cells.

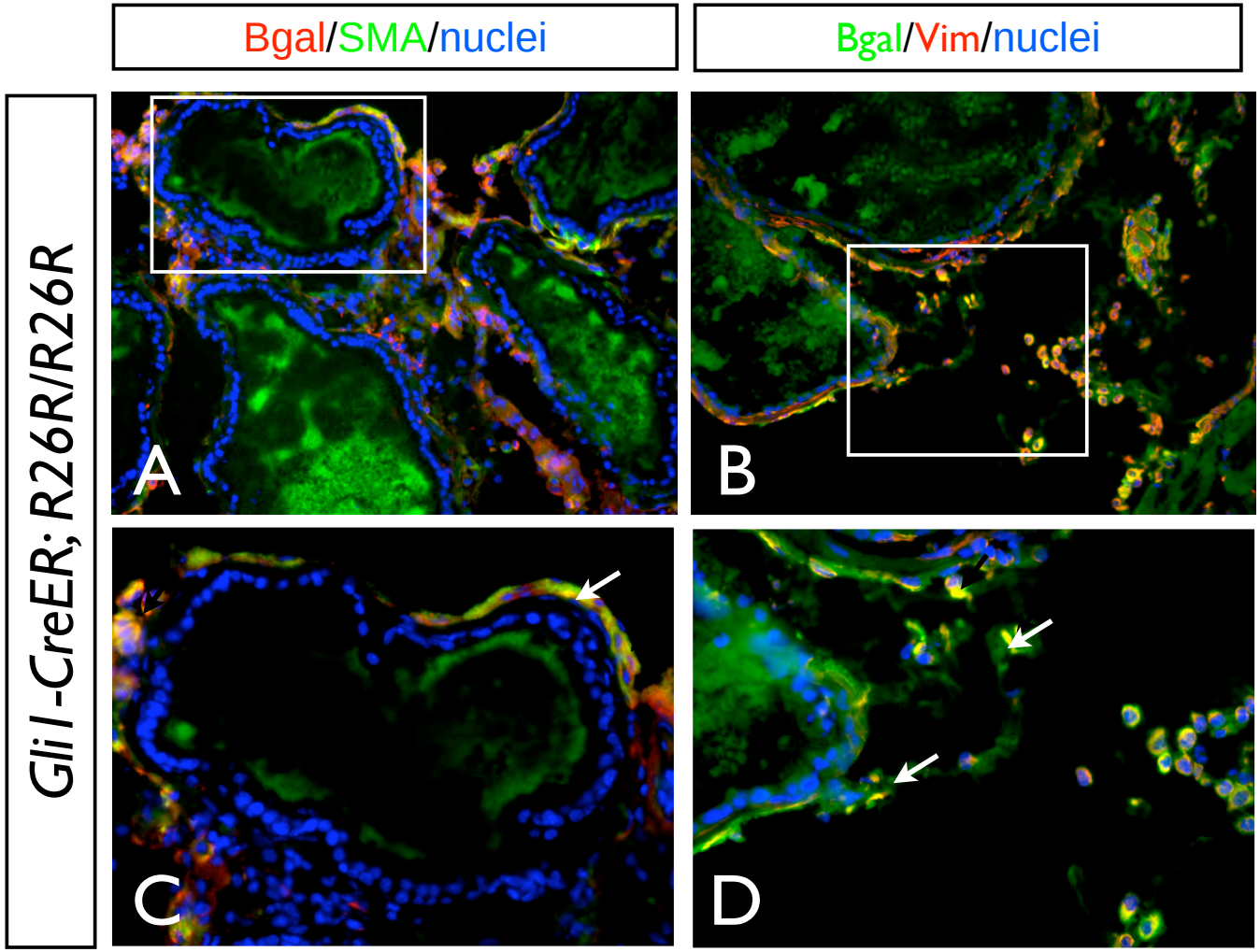

Figure 5. Fate-mapped cells after regeneration are confined to the stroma and not the epithelium. Sections from dorsal prostate of 2 month old mice, subjected to castration and susbsequent androgen treatment. Tamoxifen was administered two-weeks before castration. Mice were sacrificed two-weeks after androgen treatment, and the dorsal prostate was analyzed, using antibodies against B-gal, smooth muscle actin (smooth muscle cells) and Vimentin (fibroblasts). Marquis in A and B, indicates region magnified in C and D. Arrow in C and D, indicate double labeled cells.

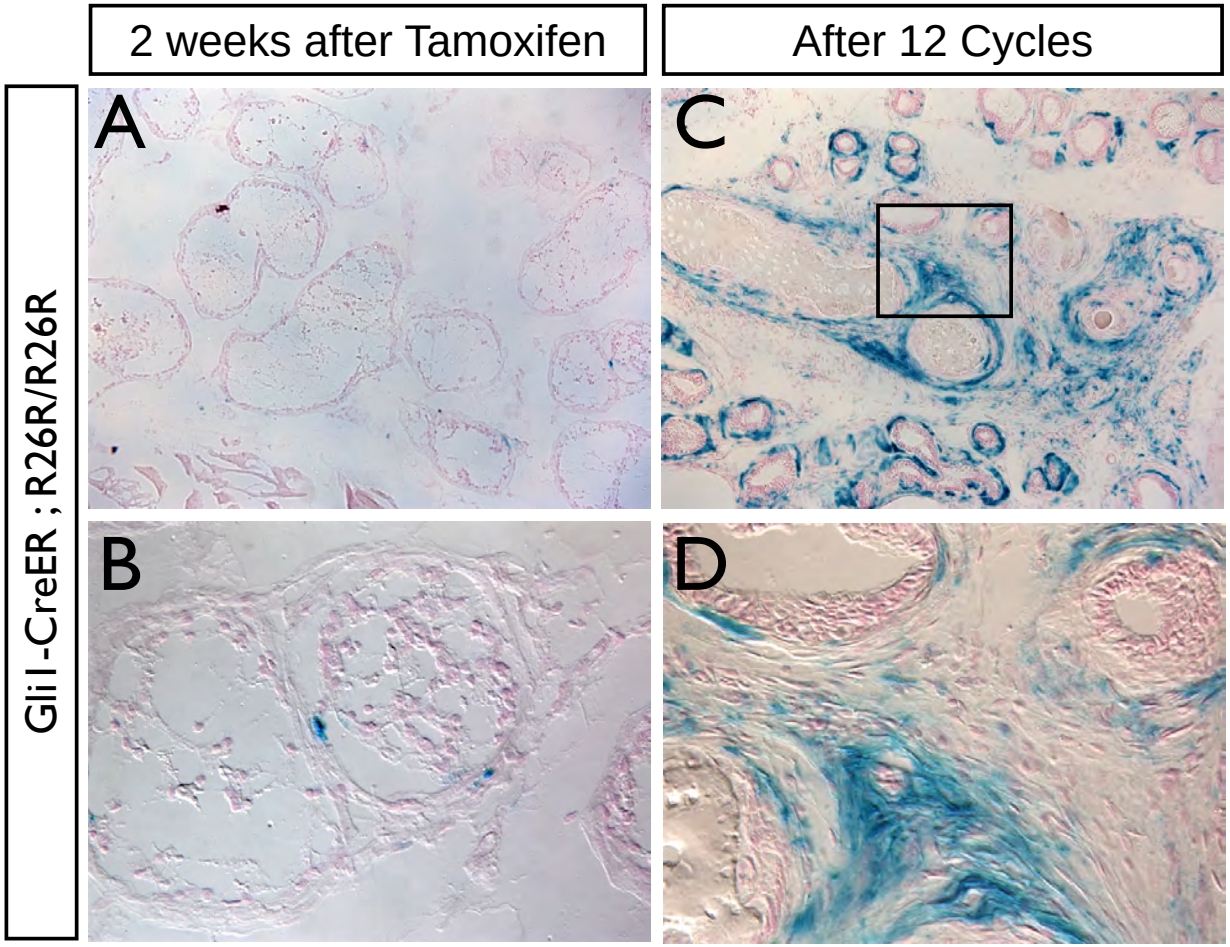

Figure 6. Gli1-expressing cells can self-renew for at least 12 cycles of involution/regeneration. *Gli1-CreER ; R26R* mice mice were given tamoxifen and castrated 2 weeks later. Following a 2 week involution, slow-release androgen pellets were implanted subcutaneously over the right shoulder. 2 weeks later, the pellets were removed. After another 2 weeks, the pellets were implanted again, and this process was repeated 11 times. The mice were then sacrificed, and sections from the dorsal prostate were analyzed by X-gal staining. B is higher magnification of A and D is higher magnification of C.

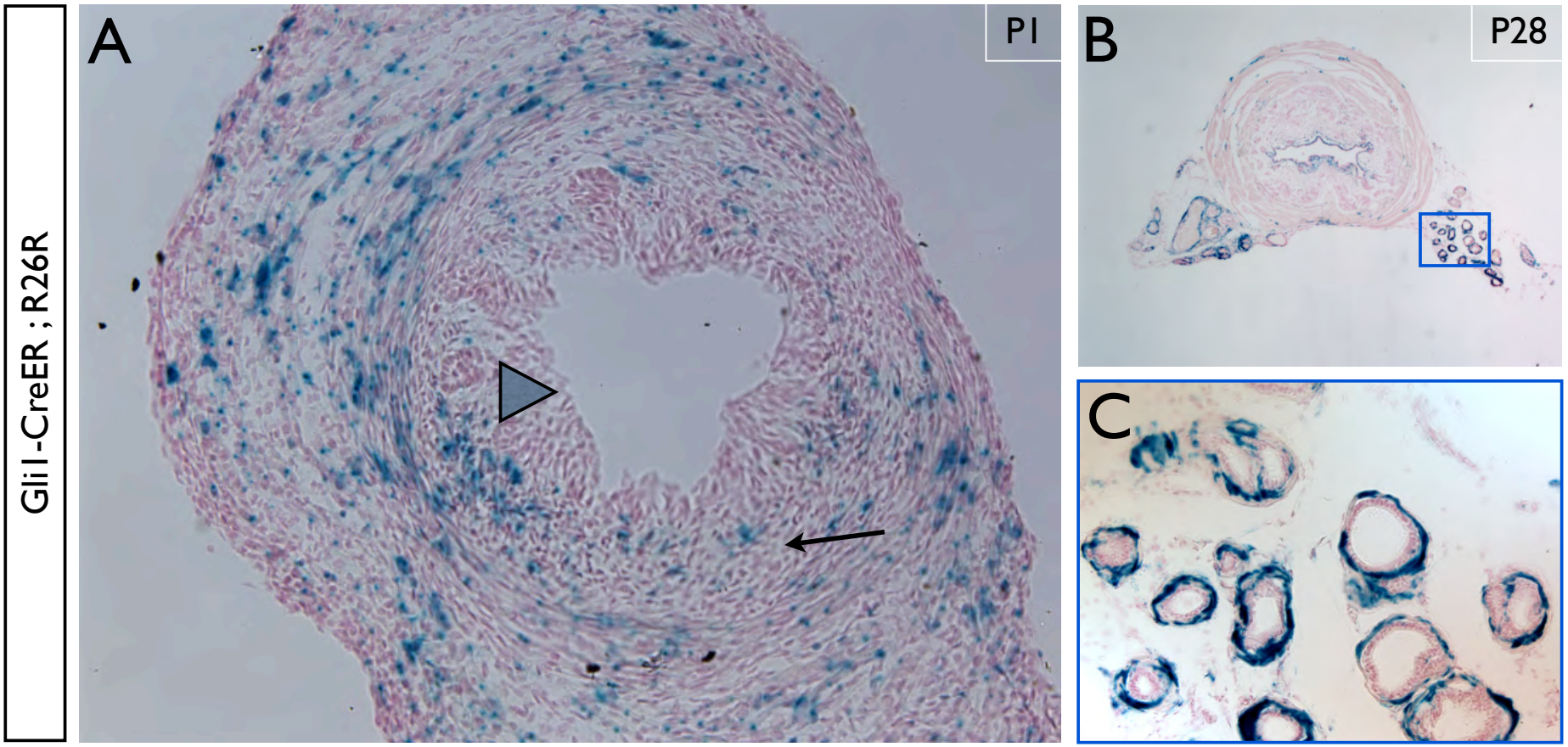

Figure 7. Gli1-derived cells marked embryonically expand after regeneration in the adult. E16.0 Gli1-CreER ; R26R embryos were given tamoxifen (pregnant mothers were gavaged) and sacrificed at P1 and at P28. Sections were analyzed by X-gal staining. Arrowhead in A indicates epithelial cells, and arrowhead indicates stroma.

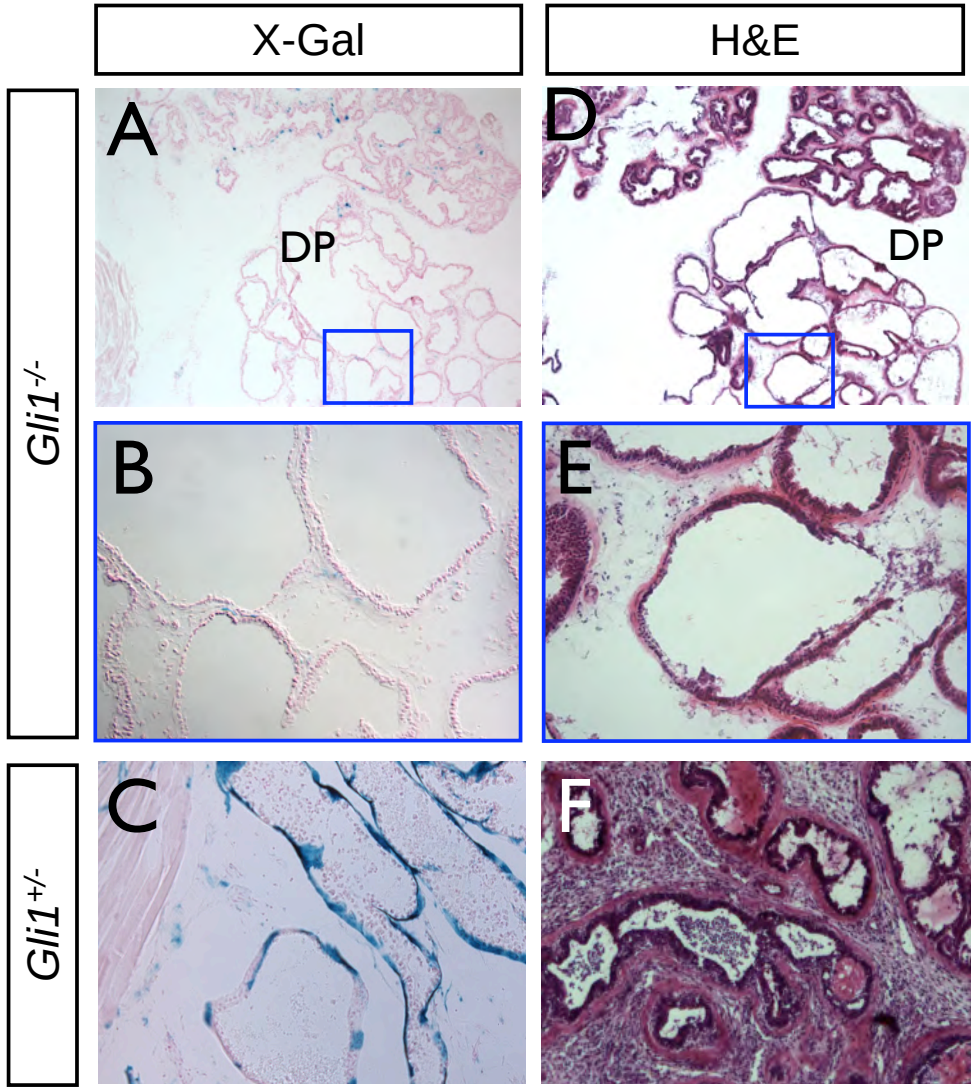

Figure 8. Stromal regeneration is impaired in *Gli1* mutants. *Gli1-CreER* homozygotes (*Gli1* mutants) were given tamoxifen, castrated 2 weeks later and subjected to one round of regeneration with androgens. A and B. X-gal stained sections from dorsal prostate. D and E. H&E stained adjacent sections from the same animals as in A and B. C. X-gal stained section from wildtype animal (Gli1-CreER/+). F. H&E stained section from WT dorsal prostate.